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Tumour necrosis factor-alpha and leukotriene B_4 mediate the neutrophil migration in immune inflammation

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1 We investigated the mediators responsible for neutrophil migration induced by ovalbumin (OVA) in immunized mice and the mechanisms involved in their release.

2 OVA administration promoted dose- and time-dependent neutrophil migration in immunized, but not in non-immunized mice, which was mediated by leukotriene B_4 (LTB_4) and tumour necrosis factor (TNF)α, since it was inhibited by LTB_4 synthesis inhibitor (MK 886) or by LTB_4 receptor antagonist (CP 105,696), by dexamethasone and by antiserum to TNFα (82, 85, 63 and 87%, respectively). Confirming TNFα involvement, OVA challenge in immunized p55 TNF receptor deficient mice ($p55^{-/-}$) did not promote neutrophil migration (control: 2.90 ± 0.68; $p55^{-/-}$: $0.92 \pm 0.23 \times 10^6$ neutrophils cavity^{-1}).

3 OVA-stimulated peritoneal cells from immunized mice released a neutrophil chemotactic factor which mimicked, in naive mice, neutrophil migration induced by OVA.

4 Supernatant chemotactic activity is due to TNFα and LTB_4, since its release was inhibited by MK 886 (93%) and dexamethasone (90%), and significant amounts of these mediators were detected.

5 TNFα and LTB_4 released by OVA challenge seem to act through a sequential mechanism, since MK 886 inhibited (88%) neutrophil migration induced by TNFα. Moreover, peritoneal cells stimulated with TNFα released LTB_4.

6 CD_4^+ T cells are responsible for TNFα release, because the depletion of this subset prevented the release of TNFα (control: 400 ± 25; immunized: 670 ± 40; CD_4^+ depleted: 435 ± 18 pg ml^{-1}).

7 In conclusion, neutrophil migration induced by OVA depends on TNFα released by CD_4^+ cells, which acts through an LTB_4-dependent mechanism.

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Keywords: Immune inflammation; LTB_4; TNFα; cytokines; neutrophil migration

Abbreviations: FLAP, five-lipoxygenase activating protein; fMLP, *N*-Formylmethionyl-leucyl-phenylalanine; IgG, immunoglobulin G; IL, interleukin; KLH, Keyhole Limpet Hemocyanin; LTB_4, leukotriene B_4; nitro, L-N^G-nitroarginine; OPD, o-phenylenediamine; OVA, ovalbumin; PBS, phosphate buffer saline; $p55^{-/-}$, p55 tumour necrosis factor receptor deficient mice; TNF, tumour necrosis factor

Introduction

Neutrophils are the principal cells involved in host defence against acute bacterial and fungal infections (Malech & Gallin, 1987). Although they have a protective effect, the tissue damage observed in diseases such as rheumatoid arthritis, glomerulonephritis, immune vasculitis and inflammatory bowel disease is, at least in part, a consequence of neutrophil accumulation (Haynes, 1992; Holdsworth & Bellomo, 1984; Wandall, 1985; Weissmann & Korchak, 1984). Neutrophil migration and activation during an inflammatory response results from several events. Among these, an important role has been ascribed to the release of chemoattractants by resident cells. To this end, it has been shown that during non-immune inflammation, macrophages and mast cells control neutrophil influx through the release of TNFα, chemokines and LTB_4 (Ajuebor *et al*., 1999; Echtenacher *et al*., 1996; Malaviya *et al*., 1996; Rankin *et al*., 1990).

With regard to the immune inflammatory reaction, T lymphocytes are thought to be the key cells that control the recruitment and activation of neutrophils. Thus, it has been demonstrated that T cells obtained from *Listeria*-immunized animals, when incubated with *Listeria in vitro*, release a factor that induces neutrophil migration (Czuprynski & Brown, 1987). Furthermore, the incubation of BCG-immune spleen cells with bacterial antigen induces the release of an antigen-specific factor, which induces neutrophil migration to the peritoneal cavity of normal mice. The release of this factor depends on spleen cell number and on the antigen dose (Appelberg, 1992). Similarly, our laboratory has demonstrated that T lymphocytes control the neutrophil migration induced by OVA in immunized rats (Klein *et al*., 1995).

Concerning the chemotactic mediators involved in the recruitment of neutrophils in immune inflammation, there is evidence that LTB_4, TNFα and chemokines participate in the recruitment induced by OVA in immunized mice (Das *et al*., 1999; Knott *et al*., 2001; Zhang *et al*., 1992; Zuany-Amorim

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et al., 1995). Furthermore, the presence of these mediators has also been demonstrated in human inflammatory bowel disease and rheumatoid arthritis, diseases in which neutrophil influx is observed (Beck & Wallace, 1997; Davidson *et al*., 1983; Edwards & Hallett, 1997; Sharon & Stenson, 1984). However, the mechanisms involved in the release and action of these mediators have not been elucidated. In the present study, we used a model of hypersensitivity reaction to evaluate the individual contributions of TNFα and LTB_4 to neutrophil recruitment, as well as the mechanisms involved in their release and action. Based on our results, we suggest that neutrophil accumulation induced by OVA in immunized mice is mediated by LTB_4, the release of which is stimulated, in turn, by TNFα produced by OVA-stimulated CD_4^+ T cells.

Methods

Animals and procedures for active sensitization

All experiments were conducted in accordance with NIH guidelines on the welfare of experimental animals. Male BALB/c, C57BL/6 and $p55^{-/-}$ mice (18–22 g) were bred and maintained in microisolator cages in the animal housing facility of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil. Breeding pairs of mice with targeted disruption of the TNFα receptor $p55^{-/-}$ gene were obtained from Jackson Laboratories (Bar Harbor, Maine, U.S.A.). Breeding stock backcrossed to C57BL/6 were obtained and the genotype of $p55^{-/-}$ mice determined by PCR of DNA as previously described (Pfeffer *et al*., 1993).

On day 0, the animals received a single s.c. injection of OVA (100 μg) in 0.2 ml of an emulsion containing 0.1 ml of phosphate buffer saline (PBS) and 0.1 ml of complete Freund's Adjuvant. The animals were given booster injections of OVA on days 7 and 14, although this time accompanied by incomplete Freund's Adjuvant. Control mice were injected s.c. with 0.2 ml of an emulsion containing equal volumes of PBS and complete Freund's Adjuvant, followed by a booster containing the emulsion of PBS and incomplete Freund's Adjuvant without OVA. Twenty-one days after the initial injection, the immunized and control animals were challenged by i.p. injection of OVA or Keyhole Limpet Hemocyanin (KLH) dissolved in PBS; alternatively, animals were injected with PBS or used as a source of peritoneal cells.

Leukocyte migration

OVA, KLH (10 μg cavity^{-1}) or PBS (0.5 ml cavity^{-1}) were injected into the peritoneal cavity of OVA-immunized or control mice. OVA was injected at doses of 1, 3 or 10 μg cavity^{-1} and leukocyte migration was evaluated at 5 min, 4, 12, 24 and 48 h post injection. At the indicated times, the animals were killed and the peritoneal cavity cells were harvested by washing the cavity with 5 ml of PBS containing 1 mM EDTA. The volumes recovered were similar in all experimental groups and approximately 95% of the injected volume was recovered. Total counts were performed in a cell counter (COULTER® A^C T; Coulter Corporation; Miami, Florida, U.S.A.) and differential cell counts (200 cells total) were carried out on cytocentrifuge (Cytospin® 3; Shandon Lipshaw Inc; Pittsburgh, Pennsylvania, U.S.A.) slides stained with Rosenfeld. The results are presented as the number of neutrophils per cavity.

Cell culture

To determine whether peritoneal cells from OVA-sensitized mice released neutrophil chemotactic factor after antigen specific stimulation, peritoneal cells from control or immunized mice were harvested by washing the cavities with RPMI-1640 medium, pH 7.2. After quantification, 1×10^6 ml^{-1} cells were incubated in the absence or in the presence of OVA (1, 3 and 10 μg ml^{-1}) or KLH (10 μg ml^{-1}). After 1 h, the cells were centrifuged, resuspended in fresh medium and further incubated for 1, 3 and 6 h. The viability of the peritoneal cells after the incubation periods, assessed by Trypan Blue dye exclusion, was greater than 95%. At the end of the incubation period, the supernatants were collected and sterilized by filtration (0.22 μm membrane; Millipore, Harrow, U.K.), and injected (1 ml) i.p. in naive mice. Cell migration was quantified 4 h after the supernatant injections. Aliquots of the supernatants were also stored at −70°C for determination of TNFα and LTB_4.

Anti-inflammatory drugs

The animals were treated 1 h before OVA challenge (10 μg cavity^{-1}) with a glucocorticoid (dexamethasone; 1 mg kg^{-1}) or with an LTB_4 synthesis inhibitor (MK 886; 1 mg kg^{-1}); alternatively, animals were treated 30 min before with a cyclo-oxygenase inhibitor (indomethacin; 5 mg kg^{-1}), or a nitric oxide synthesis inhibitor (L-N^G-nitroarginine; 50 mg kg^{-1}), or an antagonist to platelet activating factor (BN 50730; 10 mg kg^{-1}), or an antagonist to LTB_4 (CP 105,696; 3 mg kg^{-1}), or an antagonist to histamine 1 (meclizine; 20 mg kg^{-1}) receptors. Dexamethasone and L-N^G-nitroarginine were dissolved in PBS, indomethacin in 0.1 M Tris, pH 8.0, and MK 886 was dissolved in 0.1% methyl cellulose in H_2O. Meclizine was first dissolved in Cremofor EL (BASF Aktiengesellschaft, Germany), no more than 10% of the final volume, and then the volume was completed with PBS. BN 50730 and CP 105,696 were both dissolved in 10% DMSO in PBS. In some experiments recombinant murine TNFα (40 ng cavity^{-1}), LTB_4 (25 ng cavity^{-1}) or fMLP (11 μg cavity^{-1}) was injected in PBS- or MK 886-treated mice. Four hours after the challenge, neutrophil migration was assessed as described above.

The effect of anti-inflammatory drugs on the release of the neutrophil chemotactic activity by OVA-stimulated peritoneal cells was tested by adding the drugs to the incubation medium throughout the incubation period. The final concentrations used were: 1 μM for MK 886, 10 μM for dexamethasone and indomethacin and 100 μM for meclizine, BN 50730 and L-N^G-nitroarginine. The concentrations of anti-inflammatory drugs used *in vivo* and *in vitro* were as described in the literature (Bocca *et al*., 1998; Castro-Faria-neto *et al*., 1991; Jancar *et al*., 1991; Oliveira *et al*., 1994; Showell *et al*., 1995) and their effectiveness has been previously confirmed in our laboratory. To assess the possibility of dexamethasone and MK 886, present in the supernatants, causing interference in the *in vivo* neutrophil

migration assays, these substances were added to supernatants of non-pretreated OVA-stimulated peritoneal cells to give similar concentrations in each experimental group. These supernatants were assayed for neutrophil migration in naive mice.

Effect of anti-TNFα on neutrophil migration induced by OVA

The antiserum against TNFα (sheep anti-mouse TNFα: H92/B8; 35 μl cavity^{-1}) or control serum (pre-immune serum; 35 μl cavity^{-1}) was injected i.p. in sensitized mice 15 min before OVA (10 μg cavity^{-1}) challenge. Neutrophil migration was evaluated 4 h later.

ELISA

The concentrations of TNFα in the supernatants were measured by ELISA based upon a previously described protocol (Taktak *et al*., 1991). Briefly, microtiter plates were coated overnight at 4°C with an immunoaffinity-purified polyclonal sheep antibody against TNFα (2 μg ml^{-1}). After blocking the plates, recombinant murine TNFα standards at various dilutions, together with the samples, were added in duplicate at room temperature for 2 h. Rabbit biotinylated immunoaffinity-purified polyclonal anti-TNFα antibody at 1 : 1000 dilution was added, followed by incubation at room temperature for 1 h. Finally, 100 μl of avidin-HRP (1 : 5000 dilution; DAKO A/S, Denmark) was added to each well; after 30 min the plates were washed and the colour reagent OPD (40 μg well^{-1}) was added. After 15 min, the reaction was interrupted with 1 M H_2SO_4 and the O.D. was measured at 490 nm. The results were expressed as pg of TNFα ml^{-1} of the supernatant, based on a standard curve.

RIA

The LTB_4 concentration in OVA (10 μg ml^{-1})-stimulated peritoneal cell supernatant was determined by radioimmuno assay (DuPont NEN® Research Products; Boston, Massachusetts, U.S.A.) according to the manufacturer's instructions.

Cell subset depletion

To investigate which lymphocyte subset was responsible for the release of the neutrophil chemotactic factor, different subsets of lymphocytes were removed from the peritoneal cell suspension before OVA stimulation using Biomagnetic separation (Dynabeads, Dynal A.S., Oslo, Norway). The antibodies used in this study recognize only specific membrane antigens present in each leukocyte cell type (Karpati *et al*., 1991; Rameshwar *et al*., 1993; Sant, 1993). The beads coated with antibodies against Thy 1.2, B220, L3T4 and Lyt 2 were used to remove T lymphocytes, B cells, CD_4^+ and CD_8^+ T-cells, respectively. The procedures were performed in accordance with the manufacturer's instructions. The peritoneal cell suspensions, depleted of the different lymphocyte subsets, were stimulated with OVA according to the procedure described above. In some

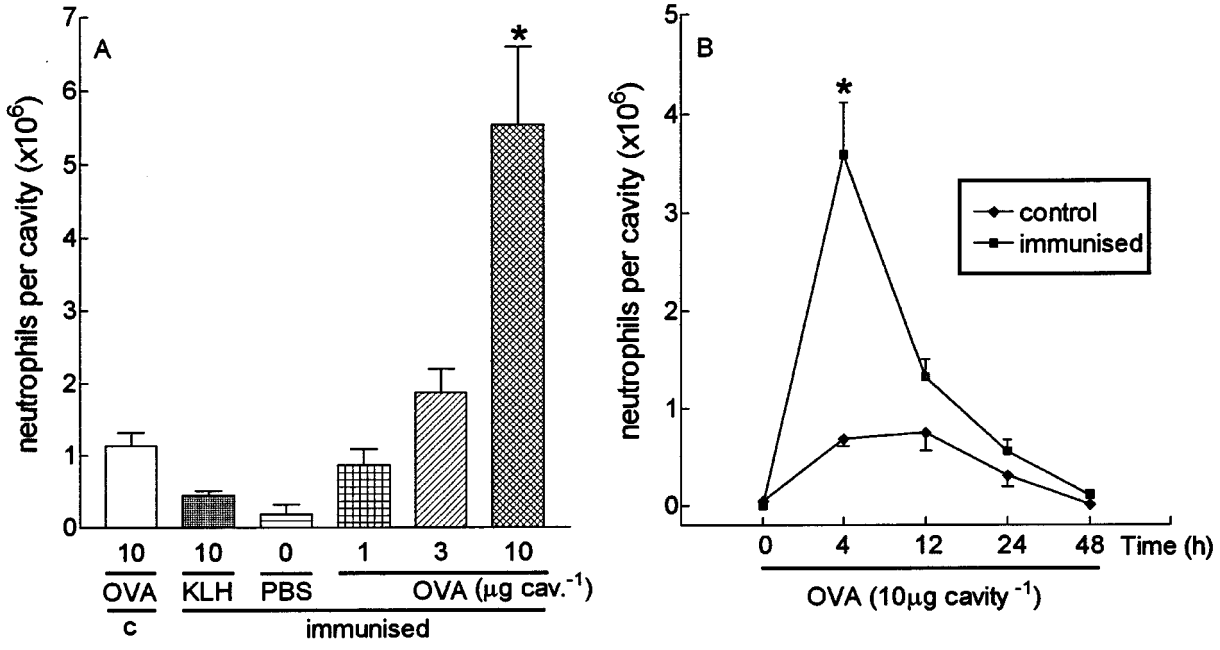

Figure 1 Dose-dependence and time-course of neutrophil migration induced by OVA. (A) OVA was injected at the indicated doses into the peritoneal cavity of non-immunized (c; control) or immunized animals and neutrophil migration was determined 4 h later. Neutrophil migration was also evaluated when 10 μg of KLH was injected into immunized mice. (B) Time-course of the neutrophil migration induced by OVA (10 μg cavity^{-1}) in control or in immunized mice. Data are mean ± s.e.mean. * $P<0.05$ compared to the respective controls (ANOVA followed by Bonferroni *t*-test). Results are representative of two separate experiments with six mice per group.

experiments, CD_4^+ T cell-depleted peritoneal cell suspension was incubated for 2 h with OVA, washed and incubated for an additional 4 h period with CD_4^+ T cells (3.5×10^5 ml^{-1}) obtained from immunized mice.

Flow cytometric analysis

In order to confirm the efficiency of the depletion method used (see previous section), the expression of CD3, CD4 and CD8 in the peritoneal cells suspensions submitted or not to biomagnetic depletion was determined by immunofluorescence analysis with specific mAbs. Peritoneal cells were first blocked with 10% normal mouse serum for 20 min at 4°C and then stained with mAb (Anti-CD3, anti-CD4 and Anti-CD8) for 30 min at 4°C. Cells were stained with the appropriate isotype-matched FITC- or PE-labelled mAb IgG, and also with control antibodies conjugated with FITC and PE (Pharmingen, Mississauga, ON, Canada), which were used as negative controls. After staining, the cells were fixed with 2% paraformaldehyde and analysed using a FACSort Becton Dickenson flow cytometer (San Jose, California, U.S.A.). The analyses were performed in the lymphocyte cell gate of the FACSort, as previously defined.

Drugs, reagents and antibodies

Recombinant murine TNFα (lot 99/532, specific activity of 200,000 IU μg^{-1} ampoule^{-1}) and the sheep anti-mouse TNFα antiserum (H92/B8) were gifts from Dr S. Poole (National Institute for Biological Standards and Control, NIBSC, London, U.K.). CP 105,696 was a gift from Professor Mauro Teixeira (Federal University of Minas Gerais, Minas Gerais, Brazil). MK 886 and indomethacin were obtained from Merck Sharp & Dohme, and BN 52021 and BN 50730 were gifts from Institut Henri Beaufour. All other reagents were purchased from Sigma.

Statistical analysis

The data are reported as mean ± s.e.mean. and are representative of two or three different experiments. The means from different treatments were compared by ANOVA. When significant differences were identified, individual comparisons were subsequently made with the Bonferroni *t*-test for unpaired values. Statistical significance was set at $P<0.05$.

Results

The i.p. injection of OVA in immunized mice induced dose-dependent neutrophil migration (Figure 1A), which peaked 4 h after the challenge and returned to control levels after 24 h (Figure 1B). The injection of OVA in immunized animals, but not in control, also induced eosinophil and mononuclear cell migration. At 24 h, when the number of neutrophils had returned to basal levels, a significant eosinophil and mononuclear cell accumulation was detected (eosinophils: control 0.01 ± 0.01; immunized 1.81 ± 0.28*, mononuclear cells: control 1.42 ± 0.52; immunized 7.52 ± 0.88* $\times 10^6$ cells cavity^{-1}, $n=6$; *$P<0.05$), and this persisted to the last time point analysed at 48 h (eosinophils: control 0.03 ± 0.01; immunized 2.78 ± 0.59*, mononuclear cells: control 1.33 ± 0.17; immunized 8.17 ± 1.18* $\times 10^6$ cells cavity^{-1}, $n=6$; *$P<0.05$). The i.p. administration of an unrelated antigen (KLH, 10 μg cavity^{-1}) in immunized animals, or OVA (10 μg cavity^{-1}) in control (non-immunized) mice, did not induce significant neutrophil infiltration at 4 h after OVA injection (Figure 1A). Furthermore, the immunization state of the animals was confirmed since, unlike non-immunized animals, immunized mice had a high titre of serum IgG against OVA (control: not detected; immunized: detected in up to 1 : 500 serum dilution).

The neutrophil migration triggered by i.p. injection of 10 μg of OVA in immunized mice was significantly inhibited by MK 886 (1 mg kg^{-1}), by CP 105,696 (3 mg kg^{-1}) or by dexamethasone (1 mg kg^{-1}). However, treatment of the animals with L-N^G-nitroarginine (50 mg kg^{-1}), BN 50730 (10 mg kg^{-1}), meclizine (20 mg kg^{-1}) or indomethacin (5 mg kg^{-1}) was ineffective in modifying neutrophil migration (Figure 2). Another PAF antagonist, BN 52021, also failed to alter the neutrophil migration evoked by OVA in immunized mice (OVA-challenge: 5.37 ± 1.29; OVA-challenge in mice treated with BN 52021: $4.39 \pm 1.50 \times 10^6$ neutrophils cavity^{-1}; $n=5$). These results suggest that LTB_4 and cytokines are involved in the neutrophil migration induced by OVA in immunized mice. It is important to point out that the results do not rule out a possible participation of CXC chemokines in the process.

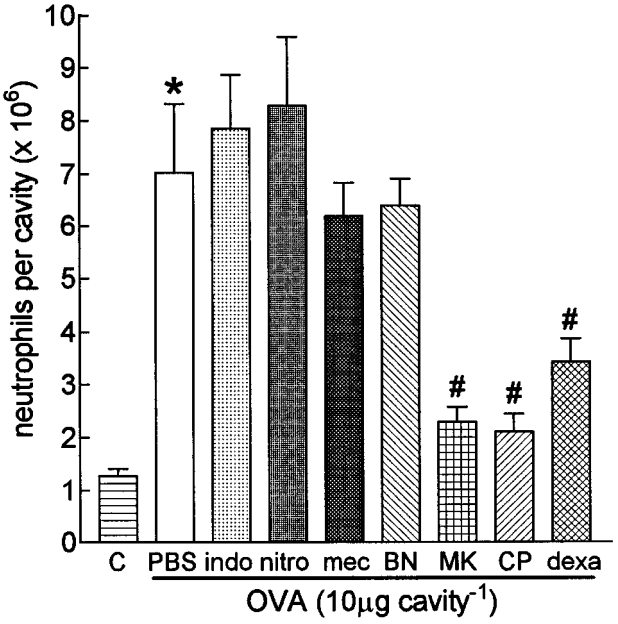

Figure 2 Effect of anti-inflammatory drugs on OVA-induced neutrophil migration. Immunized animals were treated with PBS (s.c.; 30 min before), indomethacin (indo; 5 mg kg^{-1}; s.c.; 30 min before), L-N^G-nitroarginine (nitro; 50 mg kg^{-1}; s.c.; 30 min before), meclizine (mec; 20 mg kg^{-1}; s.c; 30 min before), BN 50730 (BN; 10 mg kg^{-1}; s.c.; 30 min before), MK 886 (MK; 1 mg kg^{-1}; orally; 1 h before), CP 105,696 (CP; 3 mg kg^{-1}; s.c.; 30 min before) or dexamethasone (dexa; 1 mg kg^{-1}; s.c.; 1 h before) and then challenged with OVA (10 μg cavity^{-1}). The first bar represents the neutrophil migration induced by PBS injected i.p. (C). Neutrophil migration was evaluated 4 h after OVA challenge. The values are mean ± s.e.mean. *$P<0.05$ compared to PBS i.p. group, and #$P<0.05$ compared to PBS treated OVA-injected group (ANOVA followed by Bonferroni *t*-test) treatment. Results are representative of three separate experiments with five mice per group.

As confirmation of cytokine involvement in the neutrophil migration induced by OVA in sensitized animals, we observed that treatment of these mice with anti-mouse TNFα antiserum (35 μl) 15 min before the OVA challenge, but not with pre-immune serum, inhibited the OVA-induced neutrophil migration (Figure 3A). The same amount of antiserum against TNFα inhibited the neutrophil migration induced by i.p. administration of recombinant murine TNFα (40 ng cav.$^{-1}$) by more than 85% (TNFα i.p.: 4.25±1.06; TNFα i.p. +antiserum anti-TNFα: 0.94±0.25*×10^6 neutrophils cavity^{-1}, $n=5$; *$P<0.05$). In order to confirm the participation of TNFα in neutrophil migration in OVA-challenged immunized mice, p55$^{-/-}$ mice were actively immunized and challenged with OVA; a significant reduction of neutrophil migration was observed in these animals (Figure 3B). Meanwhile, these animals showed no significant difference from controls in their neutrophil migration response to the i.p. administration of thioglycollate (PBS: 1.1±0.4; thioglycollate in wild type mice: 11.0±0.2*; thioglycollate in p55$^{-/-}$ mice: 13.9±2.0*×10^6 neutrophils cavity^{-1}; $n=5$; *$P<0.05$ compared with PBS group).

Since we observed the participation of TNFα and LTB_4 in OVA-induced neutrophil migration, we tested the hypothesis that TNFα is inducing neutrophil migration *via* the stimulation of LTB_4 release. The i.p. administration of TNFα (40 ng cavity^{-1}) induced neutrophil migration which was inhibited by 86% after pre-treatment of mice with MK 886 (1 mg kg^{-1}). The effect of MK 886 was not nonspecific, as demonstrated by examining the effect of the same dose on the neutrophil migration induced by LTB_4 or fMLP (Figure 4)

The supernatants obtained from OVA (1–10 μg ml^{-1})-stimulated peritoneal cells (Figure 5A) from immunized mice induced a dose-related neutrophil migration into the peritoneal cavity of normal mice. On the other hand, the neutrophil migration triggered by the administration of the supernatants from OVA (10 μg ml^{-1})-stimulated peritoneal cells obtained from non-immunized mice, or from KLH (10 μg ml^{-1})-stimulated peritoneal cell supernatants obtained from immunized mice, did not differ from the neutrophil migration induced by non-stimulated peritoneal cell supernatants (Figure 5A).

The release of the chemotactic factor by OVA (10 μg ml^{-1})-stimulated peritoneal cells obtained from immunized mice was time-dependent (Figure 5B). Higher chemotactic activity was observed with the supernatant obtained after 6 h of incubation.

As shown in Figure 6, only dexamethasone (10 μM) and MK 886 (1 μM) were able to inhibit the release of the neutrophil chemotactic factor by OVA-stimulated peritoneal cells. Other drugs tested (indomethacin, 10 μM; L-N^G-nitroarginine, 100 μM; meclizine, 100 μM; BN 50730, 100 μM) were ineffective in inhibiting the release of the chemotactic factor by peritoneal cells. The absence of neutrophil migration observed after the injection of the supernatants from OVA-stimulated peritoneal cells pre-incubated with dexamethasone or MK 886 was not due to

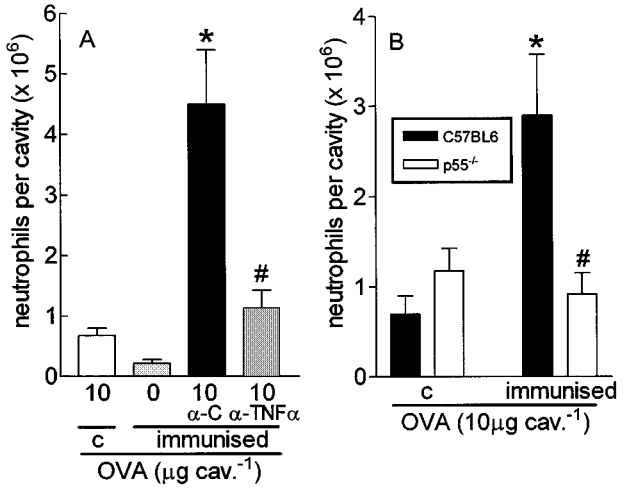

Figure 3 Inhibition of neutrophil migration by anti-TNFα serum treatment and in p55$^{-/-}$ mice. (A) BALB/c immunized mice were injected with control serum (α-C) or with anti-TNFα serum (35 μl cavity^{-1}) 15 min before the challenge with OVA (10 μg cavity^{-1}). Control (c) mice were injected with OVA and immunized mice were injected with OVA or vehicle (0.5 ml cavity^{-1}), and the neutrophil migration evaluated 4 h later. (B) Control and immunized C57BL/6 (black bars) or p55$^{-/-}$ (white bars) were challenged with OVA (10 μg cavity^{-1}), and 4 h later the neutrophil migration was estimated. Data are mean±s.e.mean. *$P<0.05$ vs non-immunized control, and #$P<0.05$ compared to α-C and immunized C57BL/6 (ANOVA followed by Bonferroni *t*-test). Results are representative of two separate experiments with five mice per group.

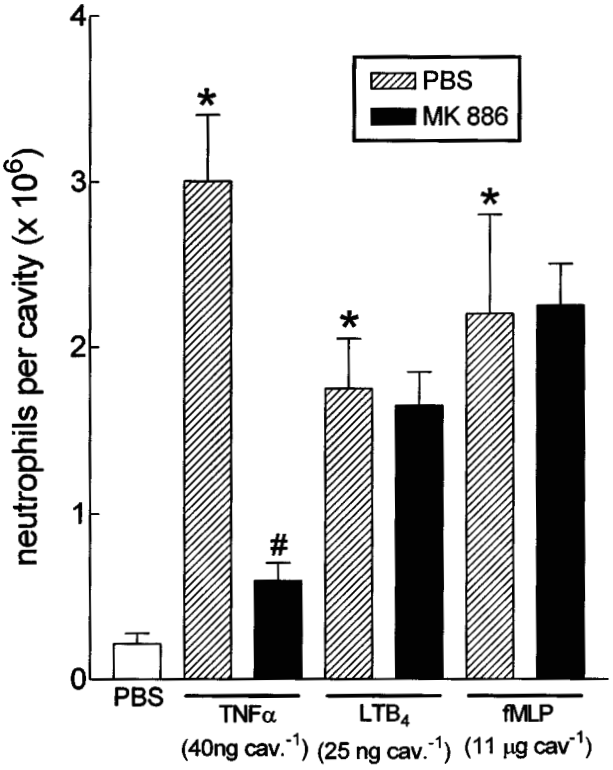

Figure 4 Effect of MK 886 on neutrophil migration induced by TNFα, LTB_4 or fMLP. Neutrophil migration was induced by TNFα (40 ng cavity^{-1}), LTB_4 (25 ng cavity^{-1}) or fMLP (11 μg cavity^{-1}) in mice pre-treated with PBS (striped bars) or MK 886 (1 mg kg^{-1}; black bar). Control mice received only PBS i.p. (white bar). Neutrophil migration was evaluated 4 h after the stimuli or PBS injection. Data are mean±s.e.mean. *$P<0.05$ compared to PBS group, and #$P<0.05$ compared to TNFα group (ANOVA followed by Bonferroni *t*-test). Results are representative of two separate experiments with five mice per group.

inhibitory effects of the drugs present in the supernatants. This is supported by the fact that the addition of dexamethasone (10 μM) or MK 886 (1 μM) to the supernatants of the OVA-stimulated peritoneal cells, at the end of the incubation period, did not interfere with their capacity to induce neutrophil migration. The migration induced by these two supernatants was 2.97 ± 0.28 and $3.15 \pm 0.31 \times 10^6$ neutrophils cavity^{-1} ($n=5$), respectively, which did not differ from the migration induced by OVA-stimulated peritoneal cell supernatant ($3.05 \pm 0.45 \times 10^6$ neutrophils cavity^{-1}; $n=6$). These *in vitro* results reinforce the participation of LTB_4 in OVA-induced neutrophil migration in the immunized mice.

The stimulation of peritoneal cells obtained from immunized mice with OVA promoted a significant increase in TNFα and LTB_4 concentrations in the supernatants compared to those observed in control supernatants (Table 1). Confirming the *in vivo* demonstration that TNFα promotes neutrophil migration *via* LTB_4, we observed that *in vitro* stimulation of peritoneal cells with TNFα (100 pg ml^{-1}) promoted a significant increase in the release of LTB_4 (medium: 114 ± 4; TNFα: 211 ± 3* pg ml^{-1} per 10^6 cells; $n=5$; *$P<0.05$).

When T cells were removed from the peritoneal cell suspension, the release of the neutrophil chemotactic factor induced by OVA stimulation was reduced, contrary to the result observed when B cells were removed (Figure 7A). Similar results were obtained when CD_4^+ T cells, but not CD_8^+ T cells, were depleted from the peritoneal cell suspension. Furthermore, the reintroduction of CD_4^+ T cells to the culture restored the release of the chemotactic factor (Figure 7B). Confirming CD_4^+ T cells as the source of TNFα, CD_4^+ T cells incubated with paraformaldehyde-fixed OVA-stimulated macrophages obtained from immunized mice released similar amounts of neutrophil chemotactic factor to those released by CD_4^+ T cells incubated with unfixed OVA-stimulated macrophages (CD_4^+ T cells + unfixed OVA-stimulated macrophages: 7.78 ± 0.42; CD_4^+ T cells + paraformaldehyde-fixed OVA-stimulated macrophages: 7.18 ± 0.53 neutrophils cavity^{-1}; $n=5$). Moreover, the supernatants obtained from cultures of CD_4^+ T cells plus OVA-stimulated macrophages from non-immunized mice induced neutrophil migration similar to that induced by CD_4^+ T cells plus macrophages from immunized mice ($6.10 \pm 0.49 \times 10^6$ neutrophils cavity^{-1}; $n=5$). Furthermore, the depletion of CD_4^+ T cells reduced by 87% the TNFα production by OVA-stimulated peritoneal cells (control: 400 ± 25; immunized: 670 ± 40; CD_4^+ depleted: 435 ± 18* pg ml^{-1}, $n=4$; *$P<0.05$). These results clearly demonstrate that CD_4^+ T cells are responsible for the TNFα release and subsequent neutrophil recruitment induced by OVA in immunized mice.

The biomagnetic depletion method used led to an efficient depletion (>98%) of the CD_4^+ T cell subset from peritoneal cell suspensions. Figure 8 depicts representative histograms for CD4 expression on peritoneal cells before and after biomagnetic depletion. The fluorescence intensity of peritoneal cells stained with anti-CD4 mAb (Figure 8B, M1 region) was dramatically reduced after CD_4^+ T cell biomagnetic

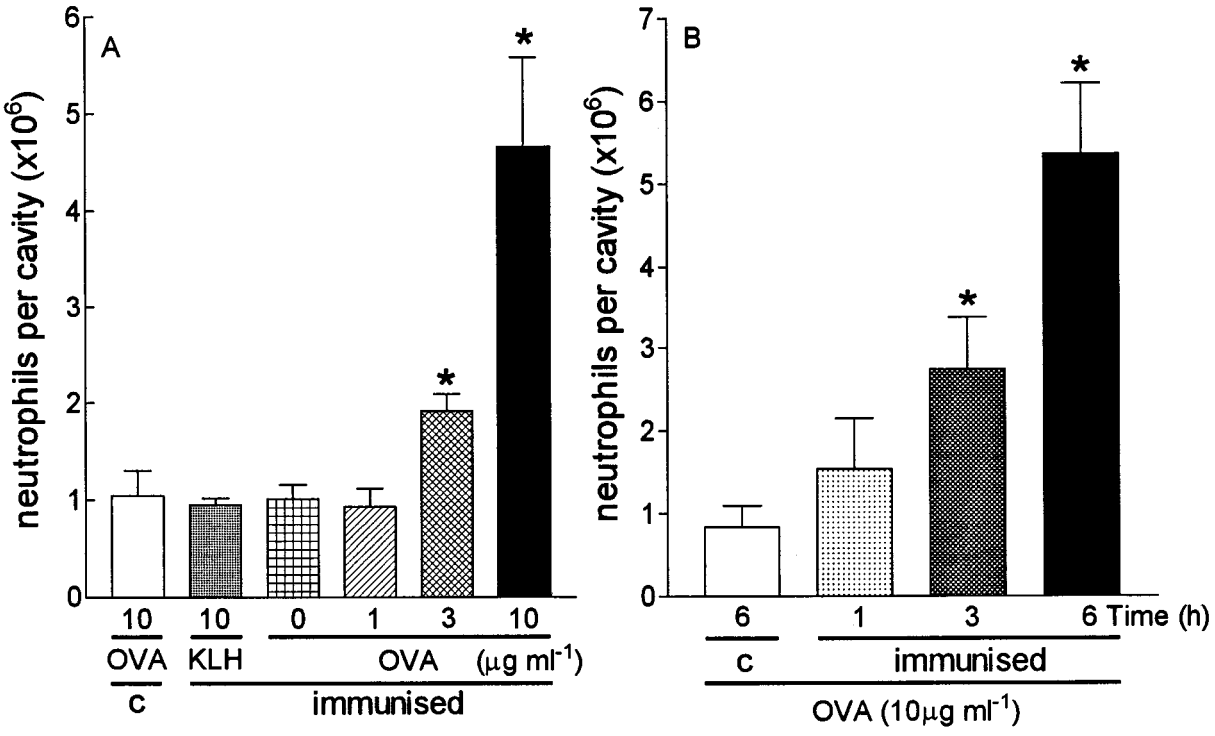

Figure 5 Release of a neutrophil chemotactic factor by OVA-stimulated peritoneal cells. (A) Neutrophil migration was induced in naive animals by the i.p. administration of 1 ml of peritoneal cell supernatant obtained from control (c) or immunized mice, which were stimulated with OVA or KLH at the indicated concentrations for 1 h, washed and cultured for 6 h. (B) Neutrophil migration induced by OVA (10 μg ml^{-1})-stimulated peritoneal cell supernatant obtained from control (c) or immunized mice, incubated for the times indicated (h). Neutrophil migration was quantified 4 h after the supernatant injections and the values are expressed as mean + s.e.mean. *$P<0.05$ compared to respective control groups (ANOVA followed by Bonferroni *t*-test). Results are representative of two separate experiments with five mice per group.

depletion (Figure 8C, M1 region). The fluorescence intensity observed in the depleted cell population was comparable to that found in peritoneal cells stained with an isotype-matched control Ab (Figure 8A, control). Peritoneal cells stained with anti-CD3 mAb exhibited the same fluorescence intensity to those labelled with anti-CD4 mAb (data not shown), thus confirming that the labelled CD_4^+ cells were lymphocytes.

Discussion

In this study we used a model of immune peritoneal inflammation to investigate the participation of TNFα and LTB_4 in the neutrophil migration in immune inflammation. We found that i.p. OVA challenge induced a dose- and time-dependent neutrophil migration in immunized mice, which peaked 4 h after challenge and returned to basal levels after 24 h, when a significant eosinophil and mononuclear cell infiltrate was observed. Similar kinetics for cell infiltration induced by antigen challenge in immunized mice and other species have been described (Abe *et al*., 1994; Klein *et al*., 1995; Metzger *et al*., 1996; Sharpe & Smith, 1979; Spicer *et al*., 1985; 1986; Zuany-Amorim *et al*., 1993). We found that OVA-induced neutrophil accumulation was dependent on endogenous release of LTB_4 and cytokines, since neutrophil recruitment was inhibited by MK 886, an LTB_4 synthesis inhibitor (five-lipoxygenase activating protein (FLAP) inhibitor; Miller *et al*., 1990), by CP 105,696, an LTB_4 receptor antagonist (Koch *et al*., 1994), and by dexamethasone, a glucocorticoid that inhibits eicosanoid and cytokine production. The results also rule out the participation of prostaglandins, nitric oxide, histamine or PAF in the process, because drugs that either inhibit their synthesis or block their receptors were ineffective in inhibiting the neutrophil migration induced by OVA. TNFα seems to be the pivotal cytokine in neutrophil influx after OVA challenge, because the treatment of immunized mice with an antibody against TNFα led to an inhibition of the neutrophil migration. In addition, significant levels of TNFα were measured in OVA-stimulated peritoneal cell supernatant. Furthermore, $p55^{-/-}$ mice immunized and challenged with OVA showed impaired neutrophil migration, compared to wild type mice, thus confirming the requirement for TNFα activity in the neutrophil migration in this model. The participation of LTB_4 and TNFα in antigen-induced neutrophil migration in immunized mice is supported by the literature. It has been shown that antigen-induced neutrophil recruitment to the peritoneal cavity of immunized mice is inhibited by an LTB_4 synthesis inhibitor, and that neutrophil accumulation in the airways of challenged, immunized mice is inhibited by anti-TNFα antiserum (Zuany-Amorim *et al*., 1993; 1995). TNFα and LTB_4 have also been found to mediate neutrophil migration in the reverse passive Arthus reaction (Steil *et al*., 1998; Zhang *et al*., 1992). Moreover, a number of studies have reported the presence of LTB_4 and TNFα in human inflammatory diseases, in which neutrophil migration occurs, such as rheumatoid arthritis and inflammatory bowel disease (Beck & Wallace, 1997; Davidson *et al*., 1983; Edwards & Hallett, 1997; Sharon & Stenson, 1984). Despite this evidence of a role for LTB_4 and TNFα in the neutrophil migration in immune inflammation the mechanisms involved in the release of these mediators, and also the mechanism by which they induce neutrophil migration, have not been clarified and were, therefore, addressed in this study.

Because both the LTB_4 receptor antagonist and the antibody against TNFα inhibited the neutrophil migration induced by OVA to a similar extent (>85%), we investigated whether recombinant TNFα might induce neutrophil migration by an LTB_4-dependent mechanism. The results suggested that recombinant TNFα-induced neutrophil migration does indeed depend on LTB_4 production since it was inhibited by MK 886 by more than 86%. This evidence for a dependence on LTB_4 production was reinforced by the observation that peritoneal cells stimulated *in vitro* with TNFα released LTB_4 into the supernatant. Moreover, TNFα-stimulated macrophages also release LTB_4 (Conti *et al*., 1989). However, in spite of the

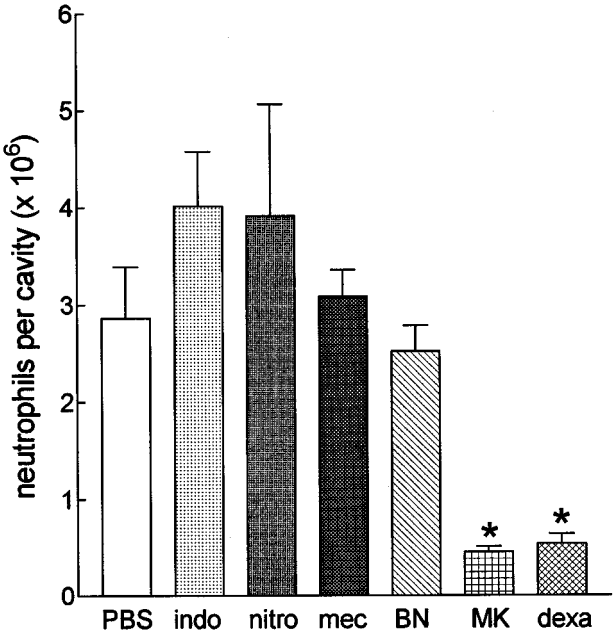

Figure 6 Effect of anti-inflammatory drugs on the release of the neutrophil chemotactic factor. Peritoneal cells were stimulated with OVA (10 μg ml^{-1}) for 1 h, washed and incubated for a further 6 h. The cells were cultured in the absence (PBS) or in the presence of indomethacin (indo; 10 μM), L-N^G-nitroarginine (nitro; 100 μM), meclizine (mec; 100 μM), BN 50730 (BN; 100 μM), MK 886 (MK; 1 μM) or dexamethasone (dexa; 10 μM). The supernatants were injected into the peritoneal cavities (1 ml) of naive mice and neutrophil migration was quantified 4 h later. Data are presented as mean±s.e.mean. $*P<0.05$ compared to the PBS treatment (ANOVA followed by Bonferroni *t*-test). Results are representative of two separate experiments with five mice per group.

Table 1 Concentration of TNF-α and LTB_4 in OVA-stimulated peritoneal cell supernatants from control or immunized mice

	Peritoneal cell supernatant	
Mediator	*Control*	*Immunized*
TNF-α (pg ml^{-1})	421±3.4	685±27*
LTB_4 (pg ml^{-1})	103.5±4.3	308.3±0.4*

Peritoneal cells (1×10^6 cells ml^{-1}) were incubated as described in the methods. Data are the mean±s.e.mean of four samples. $*p<0.05$ vs control mice (ANOVA followed by Bonferroni t-test). Results are representative of two experiments.

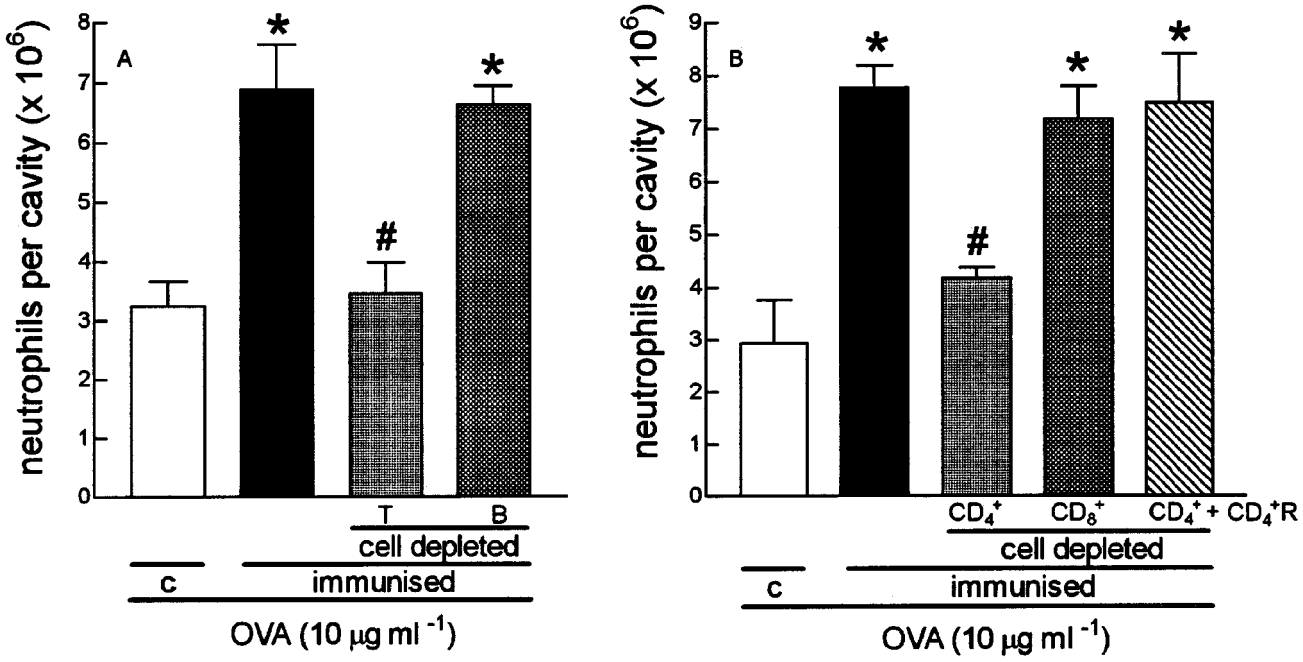

Figure 7 Effect of lymphocyte depletion on the production of neutrophil chemoattractant activity. (A) Neutrophil migration was induced in naive mice by the i.p. administration of 1 ml of supernatant of peritoneal cells obtained from control (c) or immunized mice depleted of T or B cells. (B) Neutrophil migration was induced in naive mice by the i.p. administration of 1 ml of peritoneal cell supernatant obtained from control (c) or immunized mice depleted of CD_4^+ and CD_8^+ T subsets. CD_4^+ T cell depleted peritoneal cell suspension was incubated for 2 h with OVA, washed and then reconstituted with CD_4^+ T cells (CD_4^+ R) obtained from immunized mice (striped bar). Depletion was performed as described in Methods. Data are mean ± s.e.mean. $*P<0.05$ compared to control group and $\#P<0.05$ compared to non-depleted group (ANOVA followed by Bonferroni *t*-test). Results are representative of two separate experiments with five mice per group.

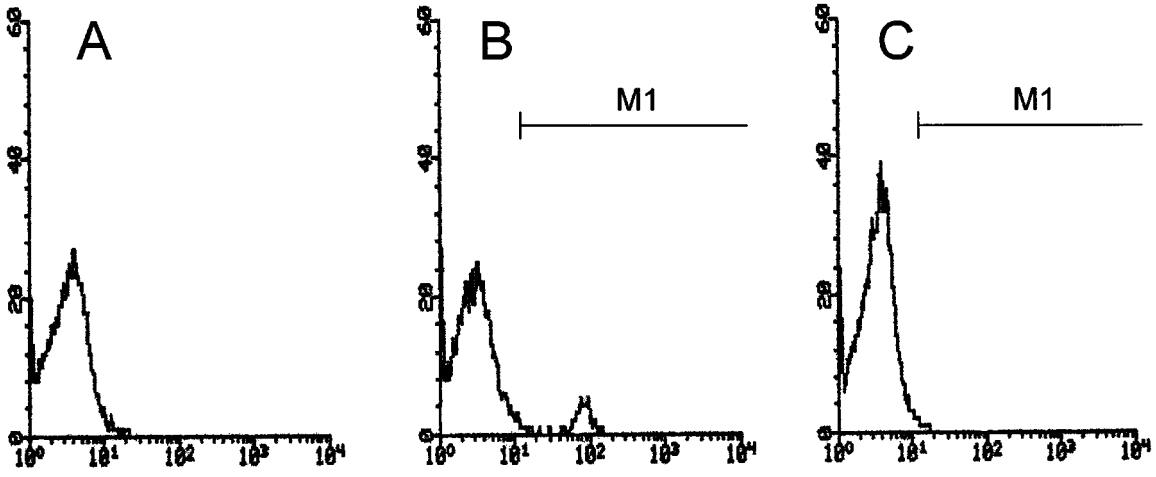

Figure 8 Representative flow cytometry histograms for CD_4 expression on peritoneal cell suspension before and after immunomagnetic depletion. Peritoneal cells were analysed for cell-surface expression of CD_4 using L3T4 FITC-conjugated monoclonal antibody. (A) Peritoneal cell suspension incubated with isotype matched antibody (control). Peritoneal cell suspension (B) or depleted CD_4^+ peritoneal cell suspension (C) were incubated with FITC-conjugated L3T4. M1 indicates the region of CD4-expressing cells. Histograms shown are from one of three separate experiments and are expressed in log scale (analysis of 10,000 cells).

demonstration that TNFα and LTB_4 are involved in neutrophil migration induced by OVA, the results do not rule out a possible participation of CXC chemokines in the process.

Next we investigated the ability of peritoneal cells obtained from sensitized animals to release a neutrophil chemotactic factor after OVA stimulation. This chemotactic factor caused, when injected in naive mice, neutrophil migration similar to that observed when OVA was injected in immunized mice. Subsequently, we confirmed that the chemotactic activity of the OVA-stimulated peritoneal cell supernatant was due to the presence of TNFα and LTB_4, since its release was inhibited by dexamethasone and MK

886. Moreover, we demonstrated significant amounts of TNFα and LTB_4 in the supernatant. We also observed that the anti-TNFα antiserum added into the culture of peritoneal cells harvested from sensitized mice 5 min before OVA-stimulation and throughout the incubation period blocked the release of the neutrophil chemotactic factor to the supernatant, which activity was evaluated in non-immune mice (data not shown).

The CD_4^+ T cells present in the peritoneal cell suspension account for the release of the TNFα involved in neutrophil recruitment, since the depletion of all T cells, or of the CD_4^+ T cell subset, prevented the release of the chemotactic factor and greatly reduced TNFα production after OVA stimulation. Moreover, the reintroduction of the CD_4^+ T cells to the culture restored the release of the neutrophil chemotactic factor. The possibility that macrophages might be a source of TNFα was excluded by incubating CD_4^+ T cells with paraformaldehyde-fixed and with unfixed OVA-stimulated macrophages; in this experiment both populations of T cells released neutrophil chemotactic factor to the same extent. The ability of CD_4^+ T cells to release TNFα has already been documented (Pawelec *et al*., 1989). However, the cell type that released *LTB*4 after stimulation by CD_4^+ T cell-derived TNFα was not determined in this study and is currently under investigation.

The release of TNFα by CD_4^+ T cells from immunized mice seems to be independent of antibodies, because it occurred even in the presence of OVA-stimulated macrophages obtained from naive animals. Moreover, the neutrophil migration was mimicked in naive mice (with undetectable OVA-specific antibodies) by the co-administration of purified CD_4^+ T cells obtained from immunized mice plus OVA-stimulated macrophages (data not shown). The pattern of response observed in this model suggests that it is representative of a hypersensitivity reaction. It is well known that this type of immune inflammation is initiated following antigen presentation in the MHC context by APC to T lymphocytes, leading to cytokine production (Gallin, 1993). In this context, our results may be important to an understanding of the neutrophil migration observed in human inflammatory disorders in which CD_4^+ T cell activation is observed, such as arthritis, inflammatory bowel disease and sarcoidosis (Deem *et al*., 1991; Panayi *et al*., 2001; Zheng *et al*., 1995). It is possible that, in such diseases, neutrophil accumulation is mediated by LTB_4, the release of which is stimulated by TNF α released by CD_4^+ T cells.

Although we did not investigate the participation of mast cells as a source of TNFα the fact that depletion of the CD_4^+ T cell subset abolished TNFα production suggests that the former cell type does not participate. The role of mast cells as a source of TNFα involved in neutrophil recruitment has been described in the reverse passive Arthus Reaction (Zhang *et al*., 1992). This discrepancy could be explained by the fact that, in the latter model, antibodies actively participate in the release of TNFα, whereas in our study immunoglobulins seem not to be involved.

Taken together, the data demonstrate that the neutrophil migration induced by OVA in immunized mice depends on TNFα release by CD_4^+ T lymphocytes. TNFα induces neutrophil recruitment *via* LTB_4, which is responsible for neutrophil recruitment into the peritoneal cavity. Therefore, inhibition of the synthesis or the actions of these two mediators could be beneficial to the control of neutrophil accumulation in inflammatory immune diseases.

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